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Cyclical changes in biomass productivity and amino acid content of freshwater macroalgae following nitrogen manipulation

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ABSTRACT

The effective supply of nitrogen to algal cultures is an important aspect of intensive cultivation and critical if the biomass is to be used as a source of protein. In this study two complementary experiments examine how variation in the supply of nitrogen to cultures influenced the biomass productivity and protein content of the freshwater macroalga, *Oedogonium*. The first examined how robust *Oedogonium* is to the intermittent supply of nitrogen (supplied weekly, every second week or every third week) by quantifying its biomass productivity, photosynthetic capacity and internal nitrogen content through time. Biomass productivity over a 12-week period was highest (10.6 g DW m^{-2} day^{-1}) when nitrogen was supplied weekly and lowest (8.1 g DW m^{-2} day^{-1}) when nitrogen was supplied every third week. The second experiment examined the recovery of nitrogen and amino acids in the biomass following periods of nitrogen-depletion. Prolonged periods (2 weeks) without nitrogen reduced the internal nitrogen and amino acid content of the biomass by up to 80%. However, in all treatments the internal nitrogen content recovered within 24–48 h and the amino acid content had recovered within 72 h following the resupply of external nitrogen. These results demonstrate that nitrogen should be supplied in a relatively constant manner to maximize the growth rates of *Oedogonium*; however, the protein in nitrogen-deplete cultures can be rapidly rejuvenated by the addition of nitrogen in the days prior to harvest.

1. Introduction

The annual consumption of protein by domesticated animals exceeds 150 million tonnes and this consumption is expected to double by 2050 [10,51]. Currently there is limited capacity to increase the production of traditional protein sources such as legumes and cereals as these crops are restricted to arable, fertile land, and require large quantities of synthetic fertilisers, many of which are finite resources themselves [21,32,47,49,50]. To overcome this future discrepancy in supply and demand, unconventional sources of protein will be required [2,10,52]. Freshwater macroalgae are a promising candidate to meet this demand as they have several advantages over existing protein crops, including the capacity to be cultivated on non-arable land and to utilize waste sources of water, nitrogen and phosphorous [15,16,26,39,54]. Under these conditions macroalgae are also capable of very high rates of biomass production, such that on an areal basis the quantity of protein produced can be up to ten times that of soybeans [16,35], which are currently the most widely used source of protein for animal feeds [10]. Furthermore, the integration of macroalgal cultivation within existing animal production industries can create a closed loop process, through which the macroalgae assimilate and convert the waste sources of nitrogen from intensive animal production into a high quality source of protein [16,54] and this macroalgae can then be used as a feed ingredient in the diets of livestock animals [18,33].

The limiting factor in the development of any new application from algae (either macroalgae or microalgae) is the reliable, intensive cultivation of biomass at large scales [20,23,35]. At the commercial, multi-hectare scale the supply of water and nutrients, primarily nitrogen, are critical to biomass productivity and the ability to balance the delivery of nutrients with the requirements for algal growth will be a major challenge [13]. In places where wastewater is rich in nutrients, one possible solution is to deliver nutrients through discrete pulses, such that the nutrients in the wastewater are effectively utilized and not lost in discharge, which occurs if cultures are maintained under constant flow [15,39,54]. A useful biological characteristic of both freshwater and marine macroalgae, which may dampen any negative effects of the intermittent supply of nutrients, is the high level of plasticity in their internal nitrogen content [5,16,39]. The internal nitrogen content of algal biomass ranges between 0.8–6.9% depending upon the amount of nitrogen available in the external environment [15,16,26,39,41] and some species can maintain high rates of growth in the short term when nitrogen supply is limited [12,27,41,45]. For example, the marine macroalga *Gracilaria tikvahiae* can maintain its growth rate for two weeks using nitrogen reserves. Moreover, the internal reserves of this species are rapidly recovered when nitrogen is re-supplied, enabling high rates of growth to be maintained [45]. In a similar manner, the fastest growth rates of the freshwater macroalga, *Oedogonium* occur

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under nitrogen limiting conditions, where internal nitrogen pools maintain growth for at least one week [15,16,41].

The ability of macroalgae to utilize their internal nitrogen reserves for growth and then rapidly replenish these reserves when external nitrogen becomes available suggests that maintaining nitrogen in excess to growth requirements may be unnecessary and could lead to reduced productivities and an increased proliferation of fouling organisms [12,24]. Furthermore, it will also result in the unnecessary release of nitrogen in the discharge water from the algal cultures [15,39,54]. For freshwater macroalgae, it is unclear whether short term increases in productivity during acute nitrogen limitation [15,16,41] can be maintained when cultures are exposed to repeated cycles of nitrogen-depletion and repletion over long periods, or whether chronic exposure to low nitrogen conditions imposes a physiological cost, such as a decline in photosynthetic capacity, resulting in lower cumulative productivity relative to cultures maintained under stable nitrogen conditions.

The nitrogen content of the macroalgal biomass and the way nitrogen is supplied to cultures, either through discrete pulses or as a continuous supply, will also have a major influence on the biochemical profile of the biomass and ultimately determine how this biomass can be used. This is particularly important for macroalgae which have applications as an ingredient in animal feeds, as the quantity and quality of protein in the biomass is closely aligned to its internal nitrogen content and, in general, an increase in the internal nitrogen content results in a corresponding increase in the concentration of the amino acids which make up proteins [4,5,16]. *Oedogonium* is an attractive source of protein for animal agriculture as it has a high proportional composition of essential amino acids [16,40], those amino acids that need to be supplied to animals through their diet [9,10,55]. Of these essential amino acids, methionine and lysine are the most important as these are generally the first-limiting amino acids for non-ruminant animals when fed plant-based diets of legumes and cereals. The amino acid content of *Oedogonium* is closely linked to its internal nitrogen content with the concentration of all amino acids (the sum of which is the protein content) declining when cultures are nitrogen-depleted [16]. While we expect the inverse of this relationship to also occur, i.e. the quantity of amino acids increases with increasing internal nitrogen content, there is limited understanding of the rate at which the concentrations of methionine, lysine and other essential amino acids recover following periods of nitrogen-depletion and repletion. This understanding will deliver a management strategy to manipulate the protein content of the algae even if the cultivation was conducted under nitrogen-limited conditions.

Therefore, the overarching aim of this study was to quantify how variation in the supply of nitrogen to cultures of *Oedogonium* affects biomass productivity, photosynthetic capacity and the rate of protein recovery. To do this we undertook two complementary studies. Firstly, we assessed the longer-term effects of the manipulation of nitrogen supply on the physiological condition and biomass productivity of *Oedogonium*. The supply of nitrogen to cultures was manipulated over a 12-week period through the pulse addition of nitrogen such that cultures underwent repeated cycles of nitrogen supply in one, two or three-weekly additions. This enabled the determination of the chronic effects of nitrogen supply on biomass productivity, internal nitrogen content and photosynthetic capacity. Secondly, we determined the time taken for the internal nitrogen and amino acid contents of *Oedogonium* to recover following periods of nitrogen-depletion of increasing duration. These experiments enabled the development of a management strategy for the supply of nitrogen for the large scale production of *Oedogonium* biomass for its targeted use as a high protein feed ingredient.

2. Methods

2.1. Study species

Oedogonium is a genus of unbranched filamentous green algae made up of small cylindrical cells. The genus has a worldwide distribution and is a common component of natural ecosystems where it is found either attached to the substrate or as free floating mats. In intensive culture conditions *Oedogonium* is a robust and competitively dominant genera that has been identified as a key target group for the bioremediation of freshwater waste streams [17,28,29,43]. The biochemical composition of *Oedogonium* males it suitable for use as a feed ingredient for animal agriculture [15,16] or for bioenergy applications [40,41]. Stock cultures of *Oedogonium* sp. TSV2 (GenBank accession number: KF606977) as described in Lawton et al. [28], and hereafter referred to as *Oedogonium*, were sourced from stock cultures maintained at the Marine & Aquaculture Research Facilities Unit (MARFU), at James Cook University (JCU), Townsville (Latitude: 19.33°S; Longitude 146.76°E). This study was conducted over a 14-week period between 1st May and 14th August 2014 and during this period the water temperature and photosynthetically active radiation (PAR) was measured daily. The daily water temperature of the tanks followed the ambient air temperature and ranged between 15.9 and 26.8 °C. The PAR was measured at the surface of cultures using a flat panel Li-190SA Quantum sensor connected to a Li-1400 data logger (Li-Cor, Lincoln, NE, USA) and the mean daily PAR was 27.2 ($\pm$0.7) mol photons m^{-2}, (range: 4.8–36.6 mol m^{-2}) with daily peaks ranging between 354 and 1870 μmol photons m^{-2} s^{-1}. These environmental conditions were consistent within an experiment and all replicates experienced the same ambient environmental conditions. No attempt was made to manipulate these conditions as they are reflective of the environment that algae will be exposed to when it is cultured at large scales.

2.2. Experiment 1: manipulation of nitrogen supply

To determine how biomass productivity, internal nitrogen content and the photosynthetic capacity of *Oedogonium* cultures are affected by the supply of nitrogen, a 12-week growth trial was undertaken in which nitrogen was supplied under three different treatments cycles of every week, every second week or every third week. This enabled the quanitification of both the immediate and longer term (chronic) effects that nitrogen supply has on the growth and photosynthetic capacity of *Oedogonium*. *Oedogonium* was cultured using nine cylindrical tanks (Duraplas AP1000; 1000 L capacity, 1.19 m^2 surface area) filled to a depth of 75 cm with dechlorinated tapwater, with a volume of ~850 L. In each of these nine tanks 250 g of fresh biomass was added to give three replicate cultures per nitrogen supply treatment. To acclimatize the biomass to the experimental tank, and to provide baseline data on the photosynthetic efficiency of *Oedogonium*, each of the nine tanks were supplied with growth nutrients (Manutec MAF; nitrogen 13.4%, phosphorous 1.4%) during the first week at a rate of 0.1 $g \cdot L^{-1}$ (85 g) added on day one. Pulse amplitude modulated (PAM) fluorometry was used to determine the potential quantum yield (Fv/Fm) of photosystem II (PSII) as a direct measurement of light stress and the photosynthetic capacity of each replicate. A high Fv/Fm ratio means that a large proportion of the incoming light energy is captured and used for photosynthesis. Fv/Fm for each treatment was measured in dark-adapted samples, using a portable PAM fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Five replicate samples from each replicate culture were placed in the fluorometer leaf-clip holder for dark-adaptation (10 min) before a saturation pulse (approximately 4000 μmol photons m^{-2} s^{-1} for 0.4 s) was applied to determine Fv/Fm. Measurements were taken at 12 pm on the day prior to harvest each week.

To determine the biomass productivity of *Oedogonium* in $g \cdot dw \cdot m^{-2} \cdot d^{-1}$, each culture was harvested weekly by draining the tank through a nylon bag. Harvested biomass was centrifuged (1000 rpm) to remove excess water and weighed to the nearest 0.1 g. Each culture was refilled with dechlorinated tapwater and restocked with fresh biomass at 0.3 $g \cdot L^{-1}$ (250 g) and, depending on the treatment cycle, nitrogen was added to these tanks through the addition of 85 g (0.1 $g \cdot L^{-1}$) of MAF nutrients. Algal productivity was calculated